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INTRODUCTION

The research activities of this Breast Cancer Center of Excellence focus on developing strategies for clinical research of breast cancer prevention, based on the role of estrogens as genotoxic agents that initiate breast and other cancers by damaging DNA. Following this underlying concept, we bring several scientific approaches to bear on investigating the ability of selected preventive agents to inhibit estrogen-induced tumors in animals and estrogen-induced mutagenesis and transformation in human breast epithelial cells. In addition, we are translating this underlying concept to humans by analyzing human breast fluid obtained by ductal lavage for estrogen metabolites, estrogen conjugates and estrogen DNA adducts, as well as expression of the genes for estrogen-metabolizing enzymes. All of the studies between the various research groups are highly interactive and synergistic. Based on the initiation of breast cancer by estrogens, this Center has a unique, innovative focus that will ultimately lead to the design of a clinical trial of breast cancer prevention.

SPECIFIC AIM 1 - CAVALIERI

The results obtained in animal models, cell culture and human breast tissue led us to select several compounds to prevent the genotoxicity of estrogens that we think is at the origin of breast cancer. The selected compounds target different steps involved in the mechanism of tumor initiation. Prevention studies will demonstrate that estrogen genotoxicity plays a critical role in the initiation of breast cancer. In addition, the results will lay the groundwork for designing a clinical research study of breast cancer prevention and developing bioassays for susceptibility to this disease. With these goals in mind, we have begun work on an animal model for breast cancer.

Specific Aim #1a: To investigate the prevention of estradiol (E_2)-induced tumors in the mammary gland of female ACI rats by analyzing the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in treated animals at various time-points and the development of tumors in the animals.

Tumor initiation studies

A study of tumor induction by E_2 and its inhibition by two antioxidants, *N*-acetylcysteine (NAcCys) and melatonin, was conducted in groups of 50 female ACI rats (9-weeks-old, Harlan, Indianapolis, IN) treated with E_2 administered by implantation of 5 mg in silastic tubing to induce mammary tumors. The groups were treated as follows: #1, E_2 ; #2, E_2 + NAcCys (low dose); #3, E_2 + melatonin (low dose); #4, E_2 + NAcCys (low dose) + melatonin (low dose); #5, E_2 + NAcCys (high dose); #6, E_2 + melatonin (high dose); #7, E_2 + NAcCys (high dose) + melatonin (high dose); #8, NAcCys (high dose) + melatonin (high dose); #9, untreated. The preventive agents were administered in the drinking water starting one week before implantation of E_2 . The rats were maintained for 6 months, monitored daily for health and tumors.

The rats were fed Teklad pellet diet. NAcCys was provided at 2 mg and 8 mg/rat/day, the low and high doses, respectively, in the drinking water at concentrations of 0.1 and 0.4 mg/ml. Melatonin was provided at 8 and 32 μ g/rat/day, the low and high doses, respectively, in the drinking water at concentrations of 0.4 and 1.6 μ g/ml.

After 2 weeks, 1 month, 2 months and 4 months, 4 rats from each group were randomly sacrificed and the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in the mammary gland are being analyzed by HPLC with electrochemical and mass spectrometric detection. In addition, the expression of selected estrogen-metabolizing enzymes was analyzed by reverse-transcription PCR. The remaining 30 rats were sacrificed at 6 months or when tumors reached a designated size, and the mammary glands were examined for tumors. The mammary tumors are being histopathologically examined by Dr. T. Seemayer, UNMC. The results will be statistically analyzed by Dr. J. Meza.

We anticipated that administration of NAcCys and/or melatonin would reduce the incidence of E₂-induced mammary tumors, which was 6.8 tumors/rat with 95% tumor-bearing animals. A reduction was not observed. In fact, no protection from tumors was achieved. While this experiment was in progress, we discovered in a related project that the mammary tissue of normal, untreated ACI rats has a number of mutations present in the H-ras oncogene (Chakravarti, et al., Proc. Amer. Assoc. Cancer Res., 44, 180, 2003). In addition, we discovered that if the E₂ implant is removed from rats having mammary tumors, the tumors regress and totally disappear within 3 to 4 weeks. Thus, this model is not a good model for testing the ability of compounds to inhibit the initiation of mammary tumors. The E₂ is presumably acting as a tumor promoter in the ACI rat mammary gland, an effect that would not lend itself to prevention.

To increase our understanding of this problem and to develop an appropriate animal model in which we can test inhibition of tumor initiation by estrogens, we are conducting a small pilot study. Groups of 10 female ACI rats (susceptible to E₂-induced mammary tumors), 10 female Sprague-Dawley rats (refractory to E₂-induced mammary tumors) and 10 male Syrian golden hamsters (susceptible to E₂-induced kidney tumors) were treated with silastic implants containing 5 mg of E₂, 5 mg of 4-OHE₂ or 5 mg of E₂ + 5 mg of 4-OHE₂. The study is currently in the 36th week. As expected, mammary tumors developed in ACI rats starting in the 19th week in the groups implanted with E₂ or 4-OHE₂ + E₂. Of particular interest is the observation that in the 29th week, new tumors began appearing in the ACI rats treated with E₂ or 4-OHE₂ + E₂, and tumors began appearing at the same time in the Sprague-Dawley rats treated with E₂ or 4-OHE₂ + E₂. At present, the E₂-treated ACI rats have 21 tumors on 9 rats and the 4-OHE₂ + E₂-treated ACI rats have 17 tumors on 7 rats. The E₂-treated Sprague-Dawley rats have 13 tumors on 5 rats, while treatment with 4-OHE₂ + E₂ has produced 18 tumors on 5 rats. We are beginning to remove the implants from half the animals with tumors to see whether these new tumors also regress. The tissues will be histologically examined. We think it is quite likely that the tumors that began appearing at 29 weeks in Sprague-Dawley and ACI rats were initiated by E₂-3,4-quinone and promoted by E₂, in contrast to the earlier tumors routinely observed in the ACI rats. Induction of these mammary tumors in Sprague-Dawley rats then should be inhibited by administration of NAcCys and/or melatonin. These tumors could be morphologically different from the ones obtained with implantation of E₂ in ACI rats and should not regress upon removal of the implant. These two parameters suggest that initiation of cancer by E₂-3,4-quinone has occurred and promotion takes place from this initiation.

Expression of estrogen-metabolizing enzymes

The expression of four estrogen-metabolizing enzymes, cytochrome P450 (CYP) 1A1, CYP1B1, catechol-O-methyltransferase (COMT) and quinone reductase (NQO1), was determined in mammary tissue collected at 2 and 4 wk after treatment with E₂ was started. Expression of CYP1A1 was also analyzed after 8 weeks of treatment. Total RNA was isolated using the TRIzol reagent. RNA was redissolved in RNase-free water, quantified by A²⁶⁰, and stored at -80°C until analysis. Absolute quantitative real-time RT-PCR was carried out using an Applied Biosystems 7700 Sequence Detection System (Applied Biosystems, Foster City, CA)

using a standard curve formed from recombinant (rc)RNA. For quantification of CYP1A1, CYP1B1, COMT, and NQO1 mRNA transcripts, PCR primers and fluorogenic probes were designed using Primer Express software (Applied Biosystems). Primers and probes were synthesized and purified by Applied Biosystems. Each tissue RNA sample was subjected to RT-PCR in two concentrations (10 ng and 1 ng) in reactions carried out using TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). Reactions were incubated at 95 °C for 10 min and then amplified using a melting temperature of 95 °C for 15 s, annealing/extension temperature of 60 °C for 1 min for 40 cycles. Control reactions to rule out contamination of RNA with genomic DNA were run by omitting reverse transcriptase from reaction mixtures that contained both rcRNA and sample RNA. To rule out other sources of contamination, control RT-PCR reactions were carried out in reaction mixtures containing no RNA. The threshold cycle of each rcRNA standard was plotted versus the log of the individual concentrations using. A trendline was added to the plot and from this the formula for the line was determined. This formula was then used to determine the value of sample RNA based on the threshold cycle. For statistical analysis, standard curves for mRNA transcript analysis were generated using Excel (Microsoft, Redmond, WA). GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to perform the Mann Whitney test to determine whether differences between groups and controls were statistically significant.

Implantation of E₂ affected the levels of all the enzymes. After 2 weeks of E₂, the mRNA expression of CYP1A1, COMT and QOR had increased approximately 7-fold. By 4 weeks, the levels were returning to normal, with COMT approximately 2-fold higher than the control, QOR less than 2-fold higher and CYP1A1 still approximately 5-fold higher than the control. By 8 weeks, CYP1A1 was less than 2-fold higher than the control. In contrast implantation of E₂ dramatically reduced the expression of CYP1B1 in the mammary tissue, with more than a 10-fold decrease by 2 weeks and greater than 50-fold decrease by 4 weeks. Administration of NAcCys and/or melatonin had no consistent effect on the expression of the selected enzymes.

These results will be correlated with the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in the mammary tissues at the various times points.

Specific Aim #1b: Analyze the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in ductal lavage samples from women with and without breast cancer.

This research project could not be started because approval to collect breast ductal lavage specimens has not yet been obtained from the Army Human Subjects Research Review Board. We have, however, made considerable progress in developing our analyses of estrogen metabolites, conjugates and DNA adducts by LC/MS, in preparation for being able to start obtaining samples of breast fluid.

Reportable Outcomes – none yet.

SPECIFIC AIM 2 - RUSSO

Introduction

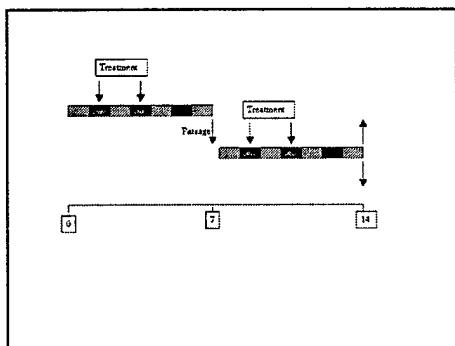
Estrogens, which are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, ER α or β [1-8]. Estrogens might act as well through alternate non-receptor mediated pathways [9-17]. If estrogen is carcinogenic in the human breast through the above mentioned pathways, it would induce transformation phenotypes indicative of neoplasia in human breast epithelial cells (HBEC) *in vitro* [18-33]. To test this hypothesis we have evaluated the transforming potential of E₂ on the spontaneously immortalized HBEC MCF-10F *in vitro* [34,35]. These cell lines lacks ER α , although ER β receptor is induced in cells transformed by chemical carcinogens [36]. The same phenotypes and characteristics expressed by MCF-10F cells transformed by the chemical carcinogen benzo[*a*]pyrene (BP) and oncogenes [37-40] were expressed in E₂-treated cells [41-54]. Thus, the MCF-10F cell line provides an excellent model to study a variety of parameters in E₂-induced transformation and mutation. In addition, the model is a powerful tool for testing preventive agents by measuring their effect on the expression of phenotypes of cell transformation and the *in vivo* expression of tumor formation in a heterologous host. **Using this model we have proposed (Aim 2 of the master application) to determine the effect of estrogen and its metabolites on the progressive steps of neoplastic transformation of human breast epithelial cells and whether the neoplastic phenotypes and genotypes thus induced can be abrogated by known and new preventive agents.**

Methods and procedures

For the purpose of our aim we have used the HBEC MCF-10F at passage 136 for treatment with different doses of E₂ or its metabolites (0.0, 0.007nm, 07nM, 7nM, 70nM, and 700nM).

Schedule of cell treatment by estrogen and its metabolites

The spontaneously immortalized HBEC MCF-10F cells were treated with estrogen or its metabolites. The steroids used include estrone (E₁), E₂, estriol (E₃), 2-OHE₁, 4-OHE₁, 2-OCH₃E₁, 4-OCH₃E₁, 2-OHE₂, 4-OHE₂, 2-OCH₃E₂ and 4-OCH₃E₂, purchased from Aldrich (Milwaukee, WI) or Steraloids (Wilton, NH). They were prepared as a stock solution of 1 mM in ethanol plus 0.1% ascorbic acid and 1% acetic acid and stored at 4 °C in the dark to prevent autoxidation of catechol estrogens (CE). Basically the cells have been treated for two periods of 24 h each. The first treatment was initiated 24 h post-plating of MCF-10F cells. The second treatment was administered after 6 days (Figure 1). This protocol has been successfully used to transform HBEC with chemical carcinogens [38, 39]. Aliquots have been collected after each treatment and after the cells have been selected in agar methocel. The data obtained will provide a direct evidence of the importance of affecting the homeostatic pathway of estrogen metabolism by measuring the transformation phenotypes *in vitro*.

**Figure 1: Schematic representation of treatment.**

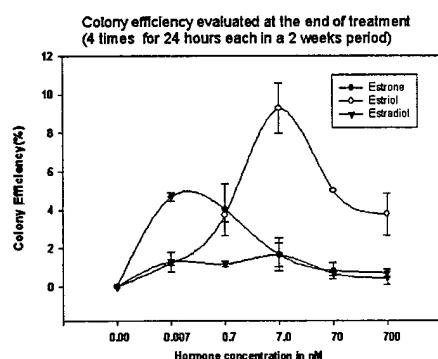
Detection of transformation phenotypes. Treated and control cells were collected and seeded at a concentration of 1×10^4 cells in 0.8% methylcellulose in 24 multi-well plates pre-coated with a layer of 0.9% agar. The cells were fed daily with fresh medium. All cultures were evaluated 24 h post-plating for detection of cell aggregates that might bias the final results, and 21 days post-plating for determination of colony efficiency as described previously [38, 39, 55, 56].

Results

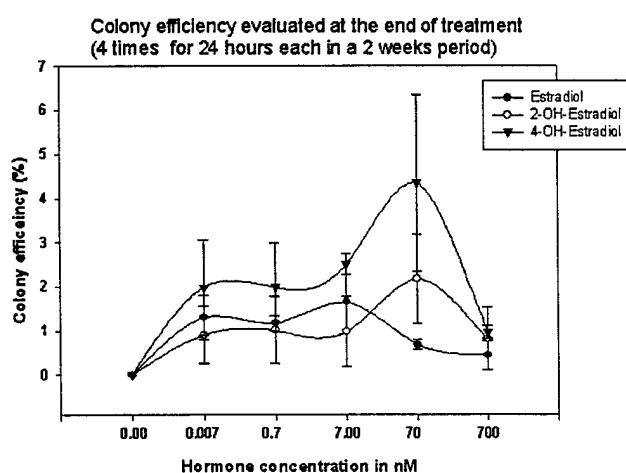
Comparative effect of Estradiol, Estrone and Estriol

The colony efficiency was determined at the end of treatment and the data are used for selecting the most efficient dose for transformation. This dose in turn is the one that will be used

for testing the chemopreventive agents *in vitro*. E_2 and E_1 are efficient in inducing colony formation in agar methocel at 0.007 nM and 0.7 nM. The sizes of the colonies were over 100 μm in diameter. A decrease in colony efficiency was detected at 70 and 700 nM. In contrast to E_2 and E_1 , the maximum colony efficiency for estriol was at 7 nM, decreasing thereafter (Figure 2).

**Figure 2: Comparative effect of E_2 , E_1 and estriol.**

Effect of catechol estrogen, 2-OHE₂ and 4-OHE₂, and the methoxy conjugated compounds.



4-OHE₂ showed an effect with 0.007 nM, whereas a higher dose (70 nM) showed an increase in colony formation. The difference was not statistically significant, although the trend was. 2-OHE₂ was less efficient than estrogen and 4-OHE₂ and needed a higher concentration to reach a significant transforming effect (70 nM) (Figure 3).

Figure 3: Comparative effect of E_2 and the two catechol forms, 2-OHE₂ and 4-OHE₂.

In Figure 4 is shown the response to colony formation in agar methocel by the 2-methoxyestradiol (2-OCH₃E₂). The curve shows higher colony efficiency with 2-OCH₃E₂ at 7

In Figure 4 is shown the response to colony formation in agar methocel by the 2-

and 70 nM, but the difference was not statistically significant. In contrast to 2-OHE₂, the 4-OHE₂ not only showed higher colony efficiency, but the methoxy conjugate (4-OCH₃E₂) was less efficient in inducing the formation of colonies, supporting the importance of inactivation of the 4-OHE₂ by methylation in the inhibition of cell transformation (Figure 5)

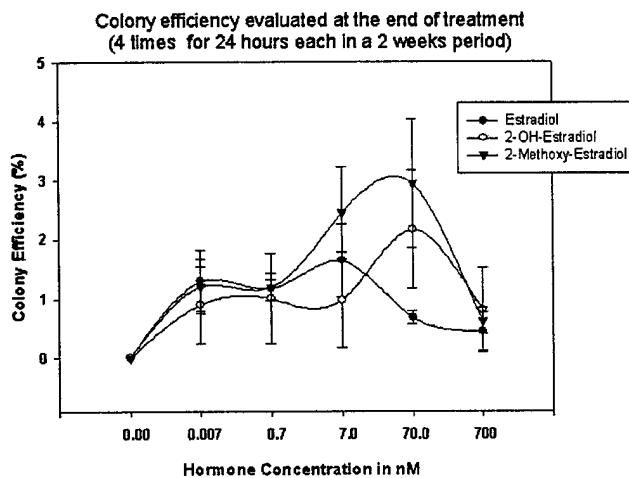
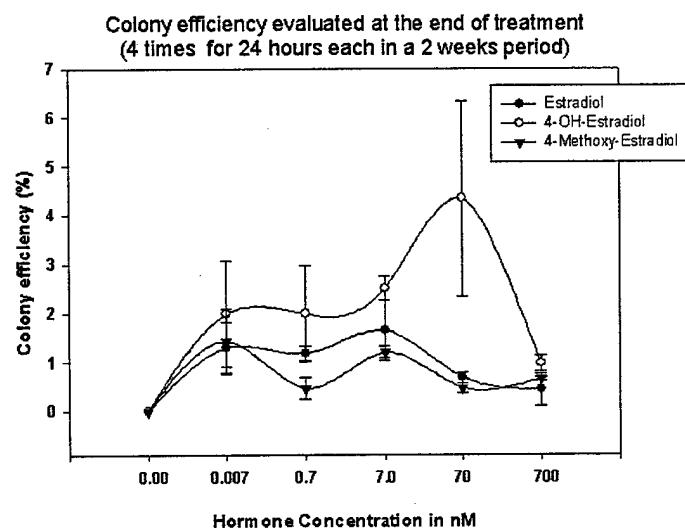
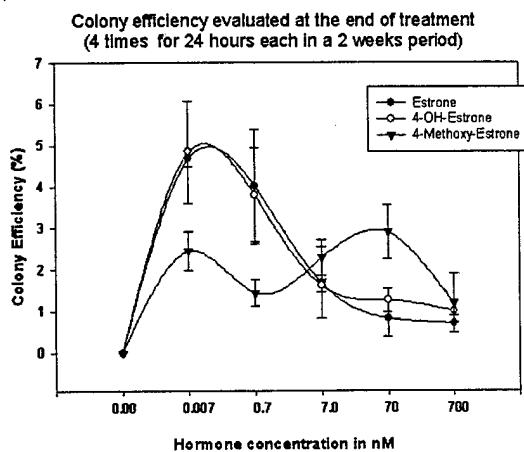


Figure 5: Comparative effect of 4-OHE₂ and the methoxy conjugate.

Figure 4: Comparative effect of 2-OHE₂ and the methoxy conjugate.



Effect of estrone, 4-OHE₁ and 4-Methoxy-estrone.



induced in human breast epithelial cells by E₂ and its metabolite 4-OHE₂.

In contrast to E₂, E₁ has a higher colony efficiency than E₂ and, interestingly, no difference was observed with 4-OHE₁, however a significant reduction in its efficiency was observed with the methoxy conjugate (Figure 6).

Figure 6: Comparative effect of E₁, 4-OHE₁ and the methoxy conjugate.

The estrogen antagonist ICI182-780 does not inhibit the proliferative activity and invasiveness induced in human breast epithelial cells by E₂ and its metabolite 4-OHE₂.

For testing whether estrogens exert their transforming effects through non-receptor mediated mechanisms, we treated the spontaneously immortalized HBEC MCF-10F, which are estrogen receptor α negative, with E_2 or its metabolite 4-OHE₂, each one either alone or in combination with the antiestrogen ICI-182-780 (ICI) for evaluating their effects on the expression of *in vitro* neoplastic transformation phenotypes. MCF-10F cells were separated into 13 groups that were treated in triplicate for two weeks as follows: 1) Control; 2) 0.007 nM E_2 ; 3) 70nM E_2 ; 4) 3.6 μ M E_2 ; 5) 0.007 nM each E_2 + ICI; 6) 70nM each E_2 + ICI; 7) 3.6 μ M each E_2 + ICI; 8) Control; 9) 0.007 nM 4-OHE₂; 10) 70nM 4-OHE₂; 11) 3.6 μ M 4-OHE₂; 12) 0.007 nM each 4-OHE₂ + ICI; 13) 70nM each 4-OHE₂ + ICI, and 14) 3.6 μ M each 4-OHE₂ + ICI. Treated cells were cultured for approximately 45 days and then evaluated for colony formation in agar-methocel (CE), tri-dimensional growth in collagen matrix, invasiveness in BioCoat Matrigel Invasion Assay, and S phase fraction determination by flow-cytometry. Both E_2 and 4-OHE₂, at all the doses tested, and in the presence or absence of ICI, increased CE and decreased the cells' ductulogenic capacity. They also increased the invasive ratios and S phase fraction of treated cells, as shown in Table 1.

Table 1-Data in invasiveness and S-phase values in MCF 10F cells treated with Estrogen and its metabolites in presence and absence of ICI-182-780

Group/ Treatment	Invasive Ratio	S Phase (%)	Group/ Treatment	Invasive Ratio	S Phase (%)
1) Control	34±13	9.96±0.93	8) Control	34± 13	9.96±0.93
2) E_2	185±5	19.93±2.08	9) 4-OHE ₂	459± 39	21.7±0.56
3) E_2	340±20	24.03±0.78	10) 4-OHE ₂	781± 61	25.6±0.52
4) E_2	617±9	20.86 ±2.02	11) 4-OHE ₂	813±120	24.2 ±3.81
5) E_2 +ICI	247±13	19.23±3.32	12) 4-OHE ₂ +ICI	522± 08	22.86 ±1.0
6) E_2 +ICI	355±54	25.93±1.0	13) 4-OHE ₂ +ICI	511.5± 27	26.60±1.4
7) E_2 +ICI	313±19	23.86 ±0.63	14) 4-OHE ₂ +ICI	776±147	22.95±5.1

Our data demonstrate that E_2 and 4-OHE₂ increase cell proliferation and induce transformation in MCF-10F cells, phenomena that are not abrogated by ICI. The failure of the antiestrogen to reverse the transformation process led us to hypothesize that estrogen-induced transformation is occurring by a non-receptor mediated process, more probably through the more potent estrogen metabolite 4-OHE₂.

Estrogen treatment of human breast epithelial cells in vitro induces loss of heterozygosity at 0.8 cM of the 13q12.3 BRCA2 locus

For investigating whether the expression of estrogen-induced transformation was associated with genomic changes, MCF-10F cells were treated with E_2 , 2-OHE₂, or 4-OHE₂ at the following doses: 0.007 nM, 70 nM, or 3.6 μ M each. DNA was extracted and analyzed by Comparative Genomic Hybridization (CGH), which revealed that only cells treated with 4-OHE₂ at the 3.6 μ M dose exhibited gain in copy number in small regions of chromosomes 9q34, 17p12-13, 17q25, 20q13, and 19p, and sporadic losses at 13q21. No aneuploidy was observed in cells treated with lower doses of 4-OHE₂ or with any of the increasing doses of E_2 and 2-OHE₂. Since

microsatellite instability (MSI) and loss of heterozygosity (LOH) in chromosome 13 have been reported in human breast carcinomas, we tested these parameters in MCF-10F cells treated with E₂, 2-OHE₂, or 4-OHE₂ alone or in combination with the antiestrogen ICI182-780 (ICI) at the same doses shown above. PCR reactions were performed by capillary electrophoresis and analyzed using the CEQ 8000 Genetic Analysis System (Beckman Coulter) using more than 10 microsatellite markers for chromosome 13. MCF-10F cells treated with all doses of E₂ and 4-OHE₂ and only with the highest dose of 2-OHE₂, either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893, which is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region. Our results indicate that estrogen and its metabolites are mutagenic in breast epithelial cells, a phenomenon that is independent of the estrogen receptor pathway.

Next proposed plan of research

To test the effect of preventive agent in cell transformation

Tamoxifen will be used to block the action of estrogens through the receptor-mediated mechanisms. D3T, a monofunctional inducer of phase 2 enzymes, and NAcCys ± vitamins C and E will also be used as preventive compounds. The estrogen compounds will be used in the physiologic range of 0.007 nM to 70 nM, at which E₂ and metabolites induced transformation of HBEC (Figs. 1-6). Dose-response curves for the preventive compounds will be established to determine the efficacious doses to be used. Treatments will be administered as indicated in Fig. 1. Basically the cells will be treated for two periods of 24 h each. The first treatment will be initiated 24 h post-plating of MCF-10F cells. The second treatment will be administered after 6 days. Aliquots will be collected after each treatment and after the cells have been selected in agar methocel. The data obtained will provide a direct evidence of the importance of affecting the homeostatic pathway of estrogen metabolism by measuring the transformation phenotypes *in vitro* and the tumorigenic response in the heterologous host.

Assays for anchorage-independent growth in agar methocel

Treated and control cells collected will be seeded at a concentration of 1x10⁴ cells in 0.8% methylcellulose in 24 multi-well plates pre-coated with a layer of 0.9% agar. The cells will be fed daily with fresh medium. All cultures will be evaluated 24 h post-plating for detection of cell aggregates that might bias the final results, and 21 days post-plating for determination of survival efficiency, colony number, colony efficiency, and colony size.

Chemotaxis and invasiveness assays

Chemotaxis and invasiveness will be determined using Boyden-type Transwell chambers (Costar, Cambridge, MA) separated by a porous polycarbonate filter (8 mm pore size) (Nucleopore, Pleasanton, CA) coated with reconstituted basement membrane material (Matrigel, Collaborative Research, Bedford, MA). Trypsinized cells will be seeded in the upper chamber and fibronectin at 1.0 µg/mL will be placed in the lower chamber as chemoattractant. The total number of cells that cross the membrane during a 12-h period of incubation will be determined under a light microscope upon fixation of the filters and staining by Diff Quick (Sigma Chemical

Co., St. Louis, MO).

Tumorigenic assay in SCID mice

The tumorigenic capabilities of transformed HBEC treated with estrogen or its metabolites will be tested by their inoculation to 45-day-old female SCID mice which will be obtained from the Fox Chase Cancer Center Animal Care Facilities. Trypsinized cells (1.0×10^7) will be suspended in 0.1 mL phosphate buffered saline and injected into the mammary fat pad of SCID mice. Tumorigenesis in these animals will be determined as described previously [38,39]. Latency period and tumor growth rate will be used as parameters of tumorigenicity.

Key research accomplishments

1-E₂, E₁, 4-OHE₂, 4-OHE₁, and, to a lesser extent 2-OHE₂ and estriol (E₃), induce phenotypes of cell transformation in HBEC treated *in vitro* at even small, or physiological doses, such as 0.007 nM.

2-E₁ was found to be more potent as a transforming agent than E₂ at equal doses. This could be a reflection of conversion of E₁ to E₂ by the enzyme 17 β -estradiol dehydrogenase.

3-The methoxy-conjugates of both E₁ and E₂ are less efficient in inducing cell transformation phenotypes, supporting the concept that conjugation of catechol is a homeostatic mechanism for reducing the genotoxic effect of these highly reactive compounds.

4-E₂ and 4-OHE₂ increase cell proliferation and induce transformation in MCF-10F cells, phenomena that are not abrogated by ICI. The failure of the antiestrogen to reverse the transformation process led us to hypothesize that estrogen-induced transformation is occurring by a non-receptor mediated process, more probably through the more potent estrogen metabolite 4-OHE₂.

5-MCF-10F cells treated with all doses of E₂ and 4-OHE₂ and only with the highest dose of 2-OHE₂, either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893. This marker is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region. Our results indicate that estrogen and its metabolites are mutagenic in breast epithelial cells, a phenomenon that is independent of the estrogen receptor pathway.

Reportable outcomes

1-Sandra V. Fernandez, Mohamed H. Lareef, Irma H. Russo, Binaifer R. Balsara, Joseph R. Testa and Jose Russo. Estrogen treatment of human breast epithelial cells *in vitro* induces loss of heterozygosity at 0.8 cM of the 13q12.3 BRCA2 locus. Proc. Am. Assoc. Cancer Res. 45:7a, 2004.

2-Mohamed H. Lareef, Rebecca C. Heulings, Patricia A. Russo, James Garber, Irma H. Russo, and Jose Russo. The estrogen antagonist ICI182-780 does not inhibit the proliferative activity

and invasiveness induced in human breast epithelial cells by estradiol and its metabolite 4-OH estradiol. Proc. Am. Assoc. Cancer Res. 45:11a, 2004.

Conclusions

During the first year of our award we have clearly demonstrated that E₂, E₁, 4-OHE₂, 4-OHE₁, and, to a lesser extent 2-OHE₂, and estriol (E₃) induce phenotypes of cell transformation in HBEC treated *in vitro* at even small, or physiological doses, such as 0.007 nM. E₁ was more potent as a transforming agent than E₂ at equal doses. This could be a reflection of conversion of E₁ to E₂ by the enzyme 17 β -estradiol dehydrogenase. The methoxy-conjugates of both E₁ and E₂ are less efficient in inducing cell transformation phenotypes, supporting the concept that conjugation of catechol is a homeostatic mechanism for reducing the genotoxic effect of these highly reactive compounds. In addition we have clearly shown that E₂ and 4-OHE₂ increase cell proliferation and induce transformation in MCF-10F cells, phenomena that are not abrogated by ICI. More importantly MCF-10F cells treated with all doses of E₂ and 4-OHE₂ and only with the highest dose of 2-OHE₂, either alone or in combination with the antiestrogen ICI, exhibited LOH in the region 13q12.3 when using the marker D13S893, which is at approximately 0.8cM upstream of the heritable breast cancer susceptibility gene BRCA2, with complete loss of one of the alleles in this region. Altogether the failure of the antiestrogen to reverse the transformation process led us to hypothesize that estrogen-induced transformation is occurring by a non-receptor mediated process, more probably through the more potent estrogen metabolite 4-OHE₂.

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SPECIFIC AIM 3 - GUTTENPLAN

This portion of the Center grant replaced Project 3 by Dr. Joachim Liehr, who unfortunately, passed away shortly after the original start date. Due to the time necessary to find the replacement project, obtain approval from subproject PI's, the DOD, collaborating institutes and IACUC committees for the replacement animal protocols, a new subcontract was issued on October 28, 2003, and rats for this subproject were first obtained in December, 2003. Thus, experiments on this revised Project 3 were begun about five months after the Center Grant start date.

The first year aims were to determine whether certain putative proximate and ultimate mutagenic metabolites of E₂ were mutagenic in the cII or lacI gene of lacI rat mammary tissue, and whether there was a difference in mutagenic activity in mammary and liver tissue. The first compounds tested were 4-OHE₂, 2-OHE₂, and E₂-3,4-quinone (E₂-3,4-Q) in addition to a vehicle control. The first two are putative proximate mutagenic metabolites of E₂ and the latter, the ultimate mutagenic metabolite. If any of the above compounds proved mutagenic, another portion of the aim was to determine its (their) mutational spectrum. Each compound was administered as an intramammary injection into each of four inguinal mammary glands of lacI transgenic Fisher (Big Blue) rats (8 rats/group). 4-OHE₂ and E₂-3,4-Q were administered at two different doses (200 and 800 nmol doses into each gland) and 2-OHE₂ at the higher dose. As the vendor only had a limited number of rats at the correct age (5-6 weeks) there was only a sufficient number of rats for a single dose of 2-OHE₂, and we chose the higher dose. An additional aim for the second half of the first year was to begin to determine the effect of potential inhibitors of mutagenesis by estrogen metabolites that proved mutagenic. Due to the late start of this project this aim will be deferred until the 02 project year.

Neither 4-OHE₂ nor E₂-3,4-Q resulted in any significant increase in mutagenesis relative to untreated controls in the cII gene at the doses administered (Table 1). The low numbers of mutants observed precluded observing relatively small increases in mutagenesis, as the SD is somewhat larger when smaller numbers of mutants are detected. There are still a number of mammary samples to analyze, but most have been completed and it seems unlikely the basic outcome will change. Therefore, we started a second group of analyses on the same tissues using an alternative assay, the lacI assay. This assay usually yields similar results as the cII assay, but may be more sensitive for certain mutagens. It is generally more time and resource consuming. However, in cases such as this it may prove more feasible if mutant fractions (and therefore numbers of mutants) are increased relative to the cII assay. Our laboratory had much previous experience in the cII assay and the lacZ assay (which cannot be performed in lacI transgenic rodents), but we had not used the lacI assay. Therefore, we had to first validate the assay in our hands. For this validation we utilized DNA from a rat embryonic lacI cell line (see below) treated with a known mutagen, N-nitroso-N-ethylurea (ENU). A near linear dose-response for mutagenesis induced by ENU was observed (Table 2). Thus, we have shown we can detect induced mutagenesis in the lacI assay and are currently reanalyzing all of the DNA samples using this endpoint. These analyses are still in progress.

During the first year of the project, results from Dr. Cavalieri's laboratory were obtained showing that chronic exposure of rats to 4-OHE₂ + E₂ via slow release from implanted silastic tubing led to the induction of mammary tumors. Therefore, it was decided to expose lacI rats to a similar treatment and measure mutagenesis. With varying treatment protocols this study requires 40 female rats and this number of rats at the correct age will not be available from the vendor until June, 2004. They are currently on order and the next study with these rats will be performed upon their arrival and acclimation.

As there is a wait of several months before the next group of lacI rats is available, we began work on a specific aim that was originally slated to begin in year 02. That aim was to determine the mutagenic activity of estrogen metabolites in a lacI embryonic rat fibroblast cell line. We purchased this line from the same vendor that sells lacI rats (Stratagene). The cells have been expanded and we are now carrying along cultures of the line. As described above we validated the lacI mutagenesis assay in this line using the known mutagen ENU. Next we plan test 4-OHE₂, 2-OHE₂, and E₂-3,4-Q for mutagenic activity in this cell line. However, it was observed in Dr. Cavalieri's laboratory that E₂-3,4-Q is relatively unstable at neutral pH, and cells should be treated at weakly acidic pH (where the compound is more stable) so it does not decompose. In order to determine the effects of pH on cell survival and growth it was necessary to carry out an assay for these effects. This assay has just been completed and the cells can withstand 2 hr at a pH of 5 - 7.4 with minimal toxicity, when measured up to six days after exposure. This represents sufficient time after dosing to observe mutagenesis. We expect to begin these experiments within the next few weeks.

Table 1. Mutant Fractions in the cII gene of lacI Rats after Treatment with Estrogen Metabolites

Group	Number of rats	Dose (nmol)	Gland	Individual MF ^a	Mean MF x 10 ⁻⁵ ±SD
E ₂ -3,4-Q	7	200	left	0.32, 0 ^b , 1.35, 0, 0.93, 0.64, 0.31	0.51 0.50
E ₂ -3,4-Q	7	800	left	2.36, 0.66, 2.69, 1.98, 0.52, 1.04, 0.13	
	5		right	<u>0.63, 0.40, 0.60, 0.79, 0.13</u>	0.85 0.87
4-OHE ₂	5	200	left	1.08, 0, 0, 0, 0	0.22 0.48
4-OHE ₂	6	800	left	0.09, 1.20, 0, 1.61, 0, 0.93	0.55 0.68
Untreated	7		left	1.22, 1.16, 0.51, 1.54, 0.25, 0.34, 0.19	0.74 0.55

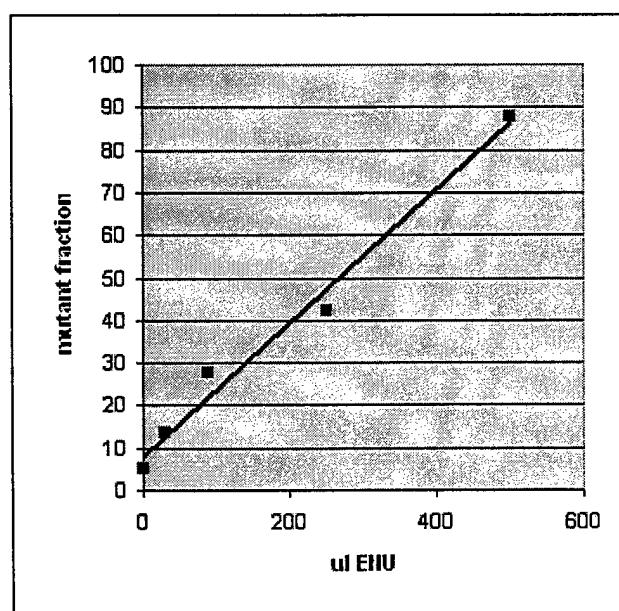
^aMF, mutant fraction = mutants/10⁵ pfu

^bA zero in the table means mutants have not yet been detected. If larger numbers of phages are screened an actual number can probably be inserted in the appropriate cells. Presumably it will be a very low number and not influence the mean mutant fraction significantly

Table 2 and plot. ENU-Induced Mutagenesis in Big Blue Rat Fibroblasts

ENU μM	MF $\times 100,000^1$
0	5.3
30	13.4
90	27.5
250	42.3
500	87.6

1. Ave of two plates

**Reportable Outcomes – none yet.**

SPECIFIC AIM 4 - SANTEN**Summary**

Our studies initially demonstrated that catechol estrogen metabolites are formed in MCF-7 human breast cancer cells in culture. Measurement involved utilization of a previously published HPLC technique with multichannel electrochemical detection. We then utilized an animal model that allows dissociation of estrogen receptor (ER) mediated function from that of the effects of E₂ metabolites. Knock-out of ER alpha in Wnt-1 transgenic mice provides a means of examining the effect of estrogen deprivation in the absence of the ER in animals with a high incidence of breast tumors. ER beta was shown to be absent in the breast tissue of these animals by RNase protection assay. In the breast tissue of these ERKO/Wnt-1 transgenic mice, we demonstrated formation of genotoxic E₂ metabolites. The ERKO/Wnt-1 breast extracts contained picomole amounts of the 4-catechol estrogens (CE), but not their methoxy conjugates nor the 2-CE and their methoxy conjugates. The 4-CE conjugates with glutathione or its hydrolytic products (cysteine and N-acetylcysteine) were detected in picomole amounts in both tumors and hyperplastic mammary tissue, demonstrating the formation of CE-3,4-quinones (Devanesan, et al., Carcinogenesis, 22, 329, 2002). These results are consistent with the hypothesis that mammary tumor development is primarily initiated by metabolism of estrogens to 4-CE and, then, to CE-3,4-quinones, which may react with DNA to induce oncogenic mutations.

The next set of experiments examined the incidence of tumors formed in Wnt-1 transgenic mice bearing wild type ER alpha (ER+/+), the heterozygous combination of genes (ER+/ER-) or both ER alpha knock out genes (ER-/-). To assess the effect of estrogens in the absence of ER, half of the animals were oophorectomized on day 15 and the other half were sham operated. Castration reduced the incidence of breast tumors in all animal groups and demonstrated the dependence of tumor formation upon estrogens. This reduction in tumor number occurred in the absence of functional ER since the number of tumors was markedly reduced in ERKO animals which were castrated early in life. In aggregate, our results support the concept that metabolites of E₂ may act in concert with ER mediated mechanisms to induce breast cancer.

We have not yet obtained data regarding the aromatase transfected animals. Final arrangements for material transfer agreement delayed the initiation of this work. We expect to begin the experiments with these animals within the next 4 months.

Methods

For the measurements of metabolites, we utilized MCF-7 cells stably transfected with the aromatase gene which our laboratory has used extensively in previous studies. In prior publications, we have described the precise methods for cell culture, counting, determination of viability, and assessment of aromatase activity in these cells.

Measurement of E₂ metabolites by the Analytical Core: The Coul-Array HPLC with 12-channel electrochemical detector method is used for measurements of specific metabolites.

Confirmation with mass spectrometry is utilized to verify the specificity of key measurements. These methods have been extensively described previously and will be only briefly described here. Samples are suspended in 50mM ammonium acetate and incubated for 6 hour with β -glucuronidase to cleave the sulfate or glucuronide conjugates. Following this step, methanol is added to bring the concentration to 60%. The mixture is then extracted with 8 ml of hexane to remove lipids. The aqueous phase is then diluted with 50mM ammonium acetate, pH 4.0, (containing 2 mg/ml ascorbic acid to minimize oxidation of catecholamines and their conjugates.) and a concentration of 25 % methanol obtained. This extract is applied to a Certify II Sep-Pak (200 mg cartridge, Varian, Palo Alto, California) and subjected to sequential elutions with 2 ml of 20 %, 40% and 70% methanol as previously described. The three fractions are analyzed by HPLC. Details of the choice of oxidation potentials, the acetonitrile/methanol/water elution gradient, the methods for peak height ratios between the dominant peak and preceding and trailing peaks in adjacent channels have been described in detail. Standards were synthesized for each of the compounds to be analyzed and used for identification and quantification of various compounds. Data analysis utilized Coul-Array software. The system is sufficiently sensitive to detect 1 pmole of metabolites and conjugates injected into the column.

ERKO/Wnt-1 and ER-/+ heterozygous mice were obtained from Dr. Kenneth Korach. Initially animals were bred from his stock of wild type Wnt-1 +/- heterozygotes. Later, we purchased the Wnt-1 wild type animals from the Jackson laboratories when our parent stock developed mouse hepatitis virus. Subsequent to reestablishing our breeding colony, all animals have tested negative for mouse hepatitis virus.

Results

MCF-7 aromatase transfected cell culture experiments

Our initial experiments examined whether the enzymes responsible for formation of depurinating metabolites were present in human breast cancer cells. Cells were incubated for 24 h with 10 μ M 4-OHE₂ before collecting media for later measurement of the various metabolites. We detected large amounts of 4 methoxy-E₂, as well as substantial amounts of the quinone conjugates and the depurinating DNA adducts, 4-OHE₁(E₂)-1-N7guanine. We next determined whether these cells could aromatize a sufficient amount of testosterone to estradiol to result in formation of the depurinating species. We detected 121 pg/ml of estrogen (118 pg/ml of E₂ and 2.89 pg/ml of E₁ in the media) indicating the production of estrogens from aromatization. The 4-OH₁(E₂)-1-N7guanine adducts were also present at a total concentration (E₁ plus E₂) of 0.92 pg/ml, as were the glutathione, cysteine, and N-acetylcysteine conjugates of E₂-3,4-Q. Finally, the aromatase inhibitor, letrozole, was capable of inhibiting the formation of the estrogens from a total of 131pg/ml of E₁ and E₂ to 2.8 pg/ml and their downstream metabolites to undetectable levels in most cases.

Measurements in ERKO/Wnt-1 mammary tissue

As previously reported, the ERKO/Wnt-1 mammary tissue appears to exhibit an altered metabolic balance. Formation of 4-OH-estrogen metabolites is favored over those of the 2-OH

species and the catechol-*O*-methyltransferase pathway appears to be relatively inactive. We detected 10.9 pmole/gm of 4-OH E₂ and E₁ in mammary tissue as well as a total of 2.3 pmole/gm of total conjugated quinone. No 4-methoxyestrogen metabolites were detected. We have not as yet been able to detect the estrogen-1-N7-guanine adduct in tissue, but are now making additional measurements on newly bred animals.

Tumor Incidence in ERKO/Wnt-1 animals

Bocchinfuso et al had previously shown that ERKO/Wnt-1 animals still exhibit a 100% incidence of mammary tumors even though ER alpha has been knocked out and they lack detectable ER beta in breast tissue. Preliminary data from this study suggested the possibility that early castration reduced the incidence of tumors and delayed the onset of tumors that did develop. To confirm and extend these results, we bred a large number of animals and performed castration on day 15 in one group. We reasoned that demonstration of a reduced incidence of tumors would provide strong support for the principle that the genotoxic metabolites of E₂ contribute to breast tumor formation. Accordingly, we castrated one group of 60 ERKO/Wnt-1⁺⁻ animals and left another group of 21 animals intact. After 12 months of observation, 50% of the intact and only 20% of the castrate animals have developed tumors. While not yet statistically significant ($p=0.2$), there is a strong trend that with time the differences will become statistically significant.

To confirm the prior results of Bocchinfuso and Korach, we also compared tumor incidence in the ER⁺⁺ Wnt-1⁺⁻ animals with those in the ER^{-/-} group. After the end of six months, 50-65% of animals in the ER⁺⁺ homozygous and ER⁺⁻ heterozygous groups had developed tumors. This is nearly identical to the 50% tumor incidence found by Bocchinfuso and Korach in these groups at 6 months. As shown previously by them, the ER^{-/-} Wnt-1 animals exhibited an incidence of only 35% at this time point.

We then extended their observations by examining the effect of early castration on tumor formation in these ER-containing animals. Castration before day 15 reduced the incidence of tumors in these two groups to 10% at six months. These data provide evidence that estradiol works both through an ER alpha dependent pathway as well as an ER independent pathway to produce breast tumors.

Reportable Outcomes – none yet.

MOLECULAR BIOLOGY CORE - SUTTER**Approved Workstatement for Year 1**

Establish DNA microarray methods with human breast tissue samples and develop and validate realtime PCR measurements for specific genes.

Progress

We have established high quality methods for DNA microarray and Realtime PCR analyses.

1) Microarray Methods

Because human breast tissue samples are extremely precious, we chose to develop methods using tissue samples of rat mammary gland.

RNA Isolation

It has been our experience that the quality of RNA is the most critical component of performing high quality microarray analysis. Therefore, we worked closely with Dr. Rogan's lab to develop methods for the rapid extraction and freezing of mammary gland tissue. On 11-07-03 RNA was isolated from 0.223 grams of rat mammary glands (received as a ground powder from Dr. Rogan). The sample was placed in a 1.5 ml tube (Axygen) containing 800 μ L Stat-60 and homogenized by passing the samples through a 23g needle on a 1ml syringe until the tissue is no longer intact. The homogenate was further processed on a Qiashredder column (Quiagen). Quality control tests were performed to assess the quality of the RNA. As shown in Table 1, the RNA yield and A260/280 ratio were satisfactory. However, as shown in Figure 1A and 1B (lane 3), the peak ratio corresponding to the 28S and 18S ribosomal RNA bands was not optimal. Specifically, the 28S band intensity should be greater than the intensity of the 18S, and this was not the case for this RNA sample, indicating minor RNA degradation.

To further improve these methods, two additional aliquots of rat mammary gland tissue were isolated and sent to the Molecular Biology Core on 11-24-03. Each sample weighed approximately 0.15 grams. The same protocol as above was used (Stat-60, needle aspiration, and Qiashredder) and quality control tests were performed. As shown in Table 2, the RNA yields and A260/280 ratios were satisfactory and improved in comparison to the first isolation. As shown in Figure 2, the quality of this RNA was high, as indicated by the appropriate ratio of the 28S and 18S ribosomal RNA bands. We were able to label this RNA and hybridize it to an Affymetrix TestChip. The results of this test indicated that this RNA is acceptable for microarray analysis.

Table 1. 11-07-03 Total RNA Concentrations and Yields for Tissue Samples of Rat Mammary Glands

Sample ID	RNA Conc. (μ g/ μ L)	Total Yield (μ g)	A 260/280
RMGT	2.777	56.00	1.7

Table 2. 11-24-04 Total RNA Concentrations and Yields for Tissue Samples of Rat Mammary Glands

Sample ID	RNA Conc. ($\mu\text{g}/\mu\text{L}$)	Total Yield (μg)	A 260/280
HB-SDR-F1 (tube 1)	2.392	69.37	1.8
HB-SDR-F1 (tube 2)	2.185	63.37	1.8

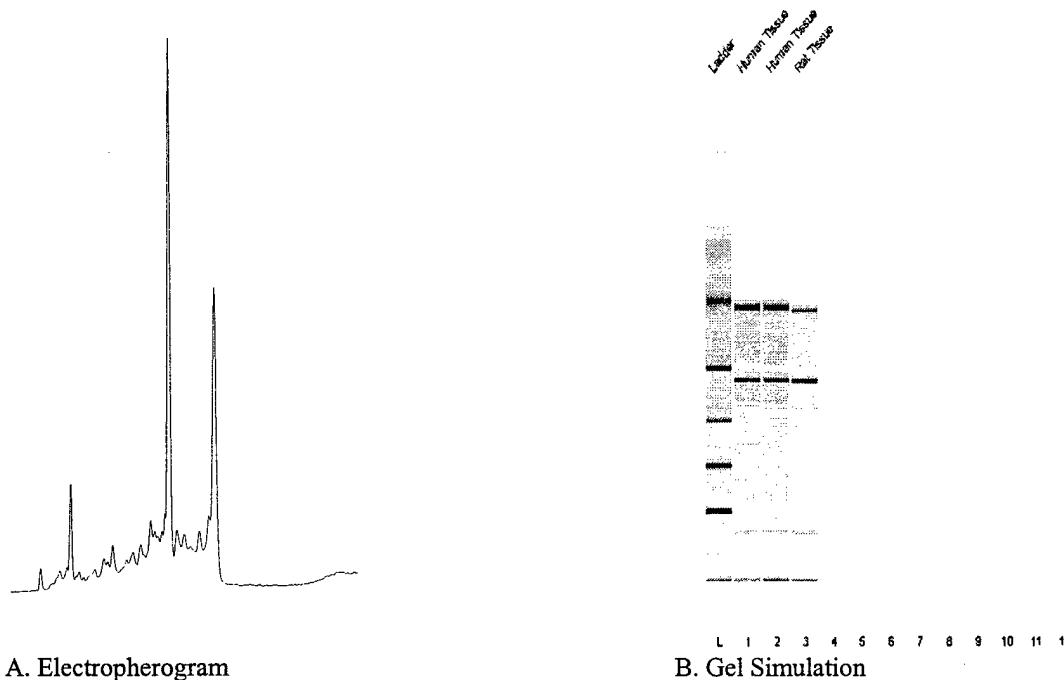


Figure 1. Capillary gel electrophoresis with electrochemical detection (Agilent Bioanalyzer 2100) of total RNA extracted from rat mammary glands (11-07-03)

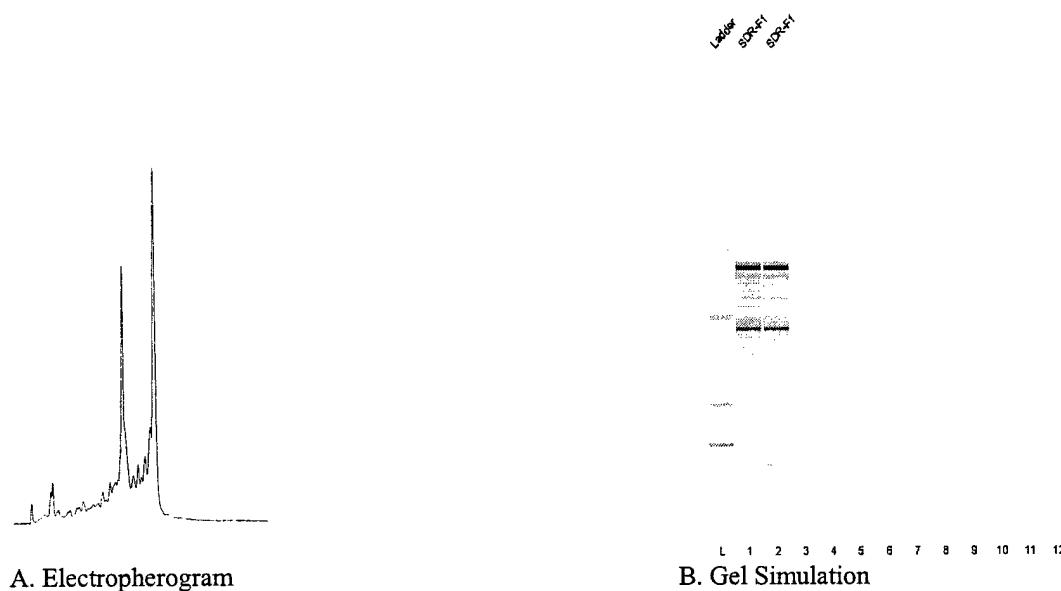


Figure 2. Capillary gel electrophoresis with electrochemical detection (Agilent Bioanalyzer 2100) of total RNA extracted from rat mammary glands (11-24-03)

Small RNA Sample Amplification and Labeling

A second major focus during this project period has been the development of methods to amplify a small amount of total RNA, in the range of 10-100 ng, in order to obtain amounts of labeled copy RNA (cRNA) sufficient for analysis of expression arrays (GeneChips). This procedure will permit analysis of total RNA isolated from as few as 100 cells. This will be especially useful in the analysis of RNA isolated from tumor tissue and neighboring normal tissue by microdissection.

The standard protocols require 5 µg of total RNA and result in yields of cRNA in the range of 20-100 µg; 18 of which is required to perform the microarray analysis. The procedure developed in our laboratory consists of two rounds of cDNA synthesis and subsequent IVT (In vitro transcription). The first IVT yields unlabeled cRNA from which the cDNA for the second IVT is synthesized. The second IVT step yields biotin labeled RNA, corresponding to the fraction of the total RNA having a poly A tail, theoretically the mRNA. In the two-cycle assay, cDNA is synthesized in the first cycle, and an unlabeled ribonucleotide mix is used in the first cycle of IVT amplification. The unlabeled cRNA is then reverse transcribed in the first-strand cDNA synthesis step of the second cycle using random primers. The T7-oligo(dT) promoter primer is used in the second-strand cDNA synthesis to generate double-stranded cDNA template containing T7 promoter sequences. The resulting double-stranded cDNA is then amplified and labeled using a biotinylated nucleotide analog/nucleotide mix in the second IVT reaction. The resulting product, the labeled cRNA is then cleaned up, fragmented, and hybridized to Genechip

expression arrays. In our pilot studies, we were able to synthesize more than 50 µg of labeled product starting with 10 ng of total RNA.

2) RealTime PCR

Methods development for RealTime PCR was based on a GeneChip study comparing the expression of genes in the livers of control or 1,2-dithiol-3-thione (D3T) treated Fisher F344 rats. Three µg of total RNA from the same batch of control and D3T treatment samples that were studied in the initial array analysis (2 each) were used for first-strand cDNA synthesis using oligo-dT and Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Gene specific primers corresponding to the PCR targets were designed using Lasergene software (DNASTAR, Inc. Madison, WI) and primers were obtained from IDT (Coralville, IA). Melt curve analysis was performed in order to check the primer specificity, and annealing temperatures were determined experimentally. Product identity was confirmed by gel electrophoresis. The PCR was performed in the Bio-Rad iCycler using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in a total volume of 50 µl. PCR master mix (25 µl) consisted of 2x Supermix (Invitrogen Life Technologies, Carlsbad, CA), 0.3 µM forward primer, 0.3 µM reverse primer, cDNA equivalent to 100 ng of RNA in a final volume of 50 µl. Amplification was carried out as follows: denaturation for 5 min at 95 °C 40 cycles of 95 °C for 30 sec, annealing for 30 sec, and 72 °C for 30 sec, with a final extension at 72 °C for 2 min. Relative gene expression was quantitated by comparative Ct method using the XLas gene with lowest coefficient of variation (CV, standard deviation /mean) from microarray data as reference gene. From the list of 246 genes that were identified as D3T-responsive, 31 genes (7 up-regulated and 24 down-regulated genes) were chosen for validation by RealTime PCR. The results of this analysis are shown in Table 3. The correlation between these two methods was greater than 0.90. In all cases, the direction of change was the same, with the a larger-fold difference observed by RealTime analysis.

TABLE 3 Validation of microarray data by real-time PCR¹

Accession No	Gene Symbol	Primer Sequence ²	Fold Change	
			Array-based	PCR-based
Upregulated Genes				
AF045464	Afar	1) 5'-CGCAGCGGCTGCAAAGTAA-3' 2) 5'-AGTGCCTGGCTGGAAAGTGTAA-3'	14.9	68.1
S72506	Gsta5	1) 5'-GAAGGTGTGGCCATCTGGAGTTG-3' 2) 5'-GCTTCTGAGGCTGGCTTTGTGG-3'	147.0	153.3
X02904	Gstp2	1) 5'-CTGGGTGCGCTTTAGGGCTTATGG-3' 2) 5'-CTTGATCTGGGGCGGGCACTG-3'	29.7	34.3
NM_133283	Map2k2	1) 5'-ATGGGGCTGTCGCTGGAG-3' 2) 5'-GGGCCGCGGTGAGACACTATGG-3'	1.7	2.5
J02679	NQO1	1) 5'-CGCGAGCGGGAAAATCT-3' 2) 5'-AATCCCCGAGGCTTGCA-3'	3.3	25.3
M31109	UDPGT	1) 5'-GGAGTTCCCATGTTCGCTGAT-3' 2) 5'-GGTCTTGGCTCCTTGTGAC-3'	1.7	7.6
AF048828	VDAC1	1) 5'-GTCGGAGTGACCCAGAGCAACTT-3' 2) 5'-GTCAGGCGAGATTGACACCAGTC-3'	2.1	2.7
Downregulated Genes				
D86086	ABCC2	1) 5'-TTCTGTCCAACGCCCTCAATA-3' 2) 5'-TTCGCTCACTGCCACAATG-3'	-1.9	-1.6

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			Cavalieri, Ercole
M74054	AGTR1A	1) 5'-TGTCTACGGCTTCTGGGAAGA-3' 2) 5'-AGGCCGTAAGAAAGCGTGCATCA-3'	-2.0 -1.9
NM_031351	ATRN	1) 5'-AGGCTGCATTGGCTTC-3' 2) 5'-GCCCTCCATCCCAGTTTAGT-3'	-1.7 -1.9
AF037072	CA3	1) 5'-TACCGACTTCGCCAATTCATCTT-3' 2) 5'-CTCGCCTTCTCCGTCTATCTT-3'	-2.6 -2.7
D14014	CCND1	1) 5'-TTGCCGCCGCTCCAGTTAG-3' 2) 5'-CAGCCACCCGCTCTCACCTC-3'	-1.9 -2.7
X75207	CCND1	1) 5'-GCGCCAGGCCAGCAGAAC-3' 2) 5'-CCGGTGGGCTCAGATGTCC-3'	-2.0 -3.0
X60769	CEPB	1) 5'-TGACACGGGACTGACGCAACAC-3' 2) 5'-AGCAACAACCCCGCAGGAACATC-3'	-1.5 -2.4
X70369	COL3A1	1) 5'-AGCCGGGCCCTCCAGAACATTAC-3' 2) 5'-CAGCTTGAAATTCTCCCTCATGG-3'	-1.8 -1.9
NM_017096	CRP	1) 5'-CCCAGGGTGCGGAAAAGTCT-3' 2) 5'-GTCAAAGCCACCGCCATACG-3'	-1.5 -3.2
K03241	CYP1A2	1) 5'-CCCCAGGAAGAGCGAGGAGAT-3' 2) 5'-GTAGCGCAGGACCGGAAAGAAGT-3'	-6.0 -2.5
D85035	DPYD	1) 5'-GGACCACTTCTGGCCCTTGTATG-3' 2) 5'-TCCGGGAAGTCAGCCTTATGTCG-3'	-2.0 -2.1
X73371	FCGR	1) 5'-GGGAGCTCCATCTGGCATCAA-3' 2) 5'-ATTCGGCACCGGTATTCTCCACT-3'	-1.7 -2.9
M91652	GLUL	1) 5'-GGAGTAGGGCGGAGTGTGAGCA-3' 2) 5'-GGTGGCCGGCAGATGATGGAT-3'	-1.8 -1.7
M11942	HSC70	1) 5'-CCCGTGGGCTCAGATTTG-3' 2) 5'-CGGCCCTGTCAATTGGTATGG-3'	-1.7 -1.9
S63521	HSPA5	1) 5'-GGGGTACCCAGATTGAAGTCACC-3' 2) 5'-GTCAGGCGGTTGGTATTGG-3'	-2.2 -2.6
NM_013060	ID2	1) 5'-GAGCAAACCCCGGTGGACGAC-3' 2) 5'-GGTGGTGCAGGCTGACGATAGTGG-3'	-1.6 -1.7
NM_013058	ID3	1) 5'-GTCGGCGGGCACTCAGC-3' 2) 5'-GGTCCAGGCGGGCTCTG-3'	-1.7 -1.8
M15481	IGF1	1) 5'-GACGGGCATTGATGAGTGTG-3' 2) 5'-CTGGGCCGGATGGAACGAG-3'	-1.7 -2.4
M31837	IGFBP3	1) 5'-TGGCCCAGCAGAAATATCAAACTC-3' 2) 5'-CTAGCCCCACCCACAACAAA-3'	-2.7 -4.1
M58587	IL6R	1) 5'-GGCGCTGAAAAAGCCAGTGATG-3' 2) 5'-TGGGGTGTGTTGCGTAACTC-3'	-1.7 -2.0
NM_134346	RAP1B	1) 5'-CCCAAGGAAAGGTCAAGAAC-3' 2) 5'-TGGCGCAACTAGTCATAAAAGA-3'	-1.6 -1.6
NM_012656	SPARC	1) 5'-TTGGGCAAGGACGCTGTGAGG-3' 2) 5'-CCGGGCTGCTGGAGTCTG-3'	-1.6 -1.2
NM_032612	STAT1	1) 5'-CACCTGCTTGCCTCTGGAAATG-3' 2) 5'-CCGGGAGCTCTACTGAACCTAAC-3'	-3.7 -10.2
X57523	TAP1	1) 5'-CGCCCCGCCCTGTAG-3' 2) 5'-GTGGCGCATGCCGTGATG-3'	-1.7 -1.7

¹Listed genes were selected from 246 D3T-responsive genes. They are major participants of carcinogen metabolism and signal transduction found in this study.

²1 indicates forward primer, 2 indicates reverse complementary primer.

Reportable Outcomes – none yet.

ANALYTICAL CORE – ROGAN

The Analytical Core provides consistent analytical power to the research projects so that estrogen metabolites, estrogen conjugates and estrogen-DNA adducts can be identified and quantified with the most sensitivity and reliability. The HPLC with multi-channel electrochemical detection enables detection of 31 metabolites and conjugates in one run. A second set-up is used exclusively to analyze the 6 catechol estrogen-DNA adducts, which need a different elution buffer to achieve separation. Our new LC/MS instrumentation has increased the sensitivity of our analyses and provides confirmation of structures.

Analytical Core

The Analytical Core uses both HPLC with electrochemical detection and LC/MS to analyze estrogen metabolites, conjugates and DNA adducts. The major effort in the Analytical Core during this year has been to work out the methods for analyzing 31 selected estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts in one LC/MS run. This work has involved determining the parent and daughter ions of the 31 compounds in the mass spectrometer and developing LC conditions to separate compounds with identical molecular weights. For example, separating 2-OHE₁ from 4-OHE₁ and 2-OHE₂ from 4-OHE₂ required considerable effort.

Medium from E₂-treated MCF-10F cells (Specific Aim 2) has been analyzed to determine the presence and levels of estrogen metabolites, conjugates and DNA adducts. These analyses are still ongoing because the level of metabolites and, thus, conjugates and DNA adducts, is very low and we are not yet satisfied with the quantitation.

Reportable Outcomes – none yet.

ADVOCACY CORE - HART

The Advocacy Core has enthusiastically embarked upon the tasks set forth in the grant's statement of work to develop a web-based model reference tool of basic scientific terminology of the Breast Cancer Center of Excellence Grant specific to the research in the grant request. The initial work has been completed in identifying a relevant article to serve as a sample - the published work of Dr. Ercole Cavalieri et al in the JNCI Monograph 27, *Estrogens as Endogenous Carcinogens in the Breast and Prostate, Chapter 4: Estrogens as Endogenous Genotoxic Agents – DNA Adducts and Mutations*. 2000. Microsoft Word has been utilized to highlight relevant scientific terms for definition to assist the layperson in reading the article and understanding the significance of the science involved. The article has been circulated to specific volunteers with rudimentary science education – i.e. basic high school biology and chemistry – to select scientific terms for which they have no understanding. These words have been defined in the context of the article by highlighting the word and inserting the definition, hidden until one clicks on the highlighted word. By hiding the definition until needed the layperson can read the scientific article in its original form with the definitions utilized as necessary. It is our belief that this serves several purposes and is the basis for the tools development.

Scientific terms are defined in the context of the scientific article so that one does not have to jump from the term to a glossary, therefore reading is easier and thought content more easily maintained.

It begins the process of familiarizing the layperson with the scientific terminology involved and the significance of the science. It thus is serving as an educational tool.

The information becomes more useful to the layperson and underscores the importance of scientific research.

The more the layperson understands the significance of scientific research, the greater the possibility of increased funding for research.

If we can iron out the glitch we have discovered in utilizing Microsoft Word, scientific articles could be written with scientific terminology defined in the context of the article, rather than always writing a lay article in addition or the layperson having to exit the article to access a glossary to look up a term. In essence, the opportunity exists to raise the level of awareness of sophisticated science in the lay community. As currently envisioned, the Word document with the highlighted terms could be placed on the Web as is and be available to read in its entirety without, as stated earlier, minimizing the article and surfing the standard glossaries in use such as the National Cancer Institute, the National Library of Medicine and a variety of other glossaries in existence.

Part II of Year 1

The Lay Guide for Advocate Participation in Basic Scientific Research has been outlined and is in the process of being written. It will be reviewed by a selected group of advocates upon completion in preparation for publication and dissemination. Currently, many advocates participate in research activities in a number of venues including the Department of Defense, National Cancer Institute, Food and Drug Administration, as well as a number of other cancer institutions, organizations, drug companies etc throughout the country. The Lay Guide being developed in this grant is specifically aimed at providing a road map for involvement in basic scientific research on an ongoing basis.

Part III of Year 1

The lay article has not been developed at this juncture as we have been occupied with the above-mentioned tasks. We have moved this to Year 2 and will begin work on this in late summer.

Advocates have attended scheduled research group meetings and have been active participants in discussions providing input from the consumer's perspective.

Reportable Outcomes – none yet.